Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in Streptomyces coelicolor A3(2)

Takano, E.; Gramajo, H.C.; Strauch, E.; White, J.; Bibb, M.J.

Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.1992.tb01459.x

Molecular Microbiology (1992) 6(19), 2797–2804

Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2)

E. Takano, H. C. Gramajo, E. Strauch,† N. Andres,‡ J. White and M. J. Bibb*
John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

Summary

Transcription of *redD*, the activator gene required for production of the red-pigmented antibiotic undecylprodigiosin by *Streptomyces coelicolor* A3(2), showed a dramatic increase during the transition from exponential to stationary phase. The increase in *redD* expression was followed by transcription of *redX*, a biosynthetic structural gene, and the appearance of the antibiotic in the mycelium, and coincided with the intracellular appearance of ppGpp. However, ppGpp production elicited either by nutritional shift-down of, or addition of serine hydroxamate to, exponentially growing cultures had no stimulatory effect on *redD* transcription. The presence of *redD* on a multicopy plasmid resulted in elevated levels of the *redD* transcript and production of *redX* and undecylprodigiosin during exponential growth; the normal growth-phase-dependent production of undecylprodigiosin appeared to be mediated entirely through the *redD* promoter, which shows limited similarity to the consensus sequence for the major class of eubacterial promoters.

Introduction

Members of the genus *Streptomyces* are notable for their ability to produce a vast array of secondary metabolites. Many of these compounds are used as antibiotics or pharmacologically active agents. In liquid medium, antibiotic production is generally limited to stationary phase, or to cultures growing at low growth rates (Demain *et al.*, 1983); in agar-grown cultures, it appears to be temporally correlated with the onset of morphological differentiation, and some genes are required for both processes, implying at least some common elements of genetic control (Chater, 1989; Hopwood, 1988). Although repression by excess phosphate or easily assimilated sources of carbon or nitrogen appears to limit production of some antibiotics (Demain, 1989), growth-rate, and potentially growth rate control, may play a general role in determining the onset of secondary metabolism. In this context, it is interesting to note the correlation, albeit incomplete, between antibiotic production and the appearance of [p]ppGpp in several streptomycetes (Ochi, 1986; 1987a,b; 1990; Strauch *et al.*, 1991; Kelly *et al.*, 1991), and the apparent ability of [p]ppGpp to reduce growth rate in *Escherichia coli* (Sarubbi *et al.*, 1988; Schreiber *et al.*, 1991).

Streptomyces coelicolor A3(2) is by far the most studied streptomycete in terms of genetics. It produces at least four chemically distinct antibiotics: actinorhodin, undecylprodigiosin, methylenomycin and a calcium-dependent antibiotic (Hopwood, 1988). We have characterized the stringent response and the production of ppGpp in this species (Strauch *et al.*, 1991); we noted a partial correlation between ppGpp synthesis and transcription of *act*III, one of the biosynthetic genes for actinorhodin (Hallam *et al.*, 1988). Here, we focus on undecylprodigiosin, a red-pigmented tripyrrole antibiotic made by the same species (the red pigmentation of *S. coelicolor* A3(2) is due to a mixture of biosynthetically related prodigionines, of which undecylprodigiosin is the major component (Tsao *et al.*, 1985); we refer to the mixture as undecylprodigiosin). Genetic studies indicate that there is a cluster of at least 18 genes (probably more) involved in undecylprodigiosin production (Rudd and Hopwood, 1980; Feitelson *et al.*, 1985; Coco *et al.*, 1991), and the entire biosynthetic cluster has been cloned on a DNA fragment of approximately 35.7 kb (Malpartida *et al.*, 1990). Towards, if not at, one end of the cluster lies a presumptive pathway-specific activator gene, *redD*. The function of this gene was predicted from the inability of *redD* mutants to co-synthesize undecylprodigiosin with other *red* mutant classes (Rudd and Hopwood, 1980; Feitelson *et al.*, 1985); from the lack of expression of the *redE* and/or *redF* genes required for *O*-methyltransferase

Received 22 April, 1992; revised and accepted 15 June, 1992. Present addresses: †Robert Koch Institut des Bundesgesundheitsamtes, Nordufer 20, 1000 Berlin 65, Germany; ‡Hoechst AG, Pharma Qualitätskontrolle Bioanalytik, 6230 Franfurt-Main, Germany. *For correspondence. Tel. (0603) 52571; Fax (0603) 56844; E-mail BIBB@UK.AC.AFRC.JII.

activity in *redD* mutants (Feitelson *et al.*, 1985); and from the ability of extra cloned copies of *redD* to elicit overproduction of undecylprodigiosin in *S. coelicolor* A3(2) (Narva and Feitelson, 1990) and *Streptomyces lividans* (Malpartida *et al.*, 1990), a close relative of *S. coelicolor* A3(2) in which little undecylprodigiosin is usually made. The nucleotide sequence of *redD* has been determined (Narva and Feitelson, 1990).

Here, we define the *in vivo redD* transcriptional start site, and study the temporal and growth-phase transcription of *redD* and *redX*, a structural gene for an early step in the biosynthetic pathway (Guthrie and Chater, 1990; Malpartida *et al.*, 1990). We assess the possible correlation between transcription of *redD* and *redX*, and the production of ppGpp, which was elicited in exponentially growing cultures either by amino acid depletion (nutritional shiftdown) or by addition of serine hydroxamate, a seryl tRNA synthetase inhibitor that induced lower levels of ppGpp. We also investigated the effect of increased levels of *redD* expression during exponential growth on *redX* transcription and on undecylprodigiosin biosynthesis.

Results

Undecylprodigiosin production in liquid culture

In minimal medium supplemented with 0.2% w/v casamino acids, *S. coelicolor* A3(2) strain M145 grew exponentially with a doubling time of 2.2 h, and made a rapid transition into stationary phase, approximately 12 h after inoculation, at an $OD_{450\ nm}$ of about 2 (approximately 1 mg ml^{-1} dry weight). Undecylprodigiosin production was readily detected as soon as the culture entered stationary phase. The low number of doublings (approximately five) limited the size of mycelial clumps, and may have served to minimize physiological heterogeneity that might have resulted from nutritional or oxygen limitation. Increasing the amount of glucose (fivefold) or phosphate (10-fold) had no effect on growth, but removal of the $(NH_4)_2SO_4$ led to a reduction in final biomass equivalent to the growth observed in nitrogen-limited minimal medium, indicating that the casamino acid-supplemented cultures were also nitrogen-limited. Supplementation with higher concentrations of casamino acids did not increase the growth rate, but did increase the final yield of mycelium. In minimal medium lacking casamino acids, M145 grew with a doubling time of about 4 h, and entered stationary phase in a less synchronous manner at an $OD_{450\ nm}$ of about 0.6; replacement of glucose with the same molar concentration of arabinose, fructose or galactose resulted in a lower growth rate and lower final $OD_{450\ nm}$ (about 0.2 for galactose and fructose). In the absence of casamino acids, undecylprodigiosin production occurred upon entry into stationary phase, regardless of the carbon source used, although the amount produced was much lower than in the presence of casamino acids. Given the more rapid transition from exponential to stationary phase, the higher levels of synthesis of undecylprodigiosin, and our desire to carry out nutritional shiftdowns, all subsequent experiments used minimal medium supplemented with 0.2% w/v casamino acids.

S1 nuclease mapping of the redD *transcriptional start site*

An RNA-protected fragment was readily detected with RNA isolated from cultures of *S. coelicolor* A3(2) M145 producing undecylprodigiosin (Fig. 1A). Transcripts corresponding in size and orientation to this apparent promoter, and to the four oppositely orientated promoters (pr1–4) identified by Narva and Feitelson (1990), were also observed in *in vitro* transcription studies using the *Nae*I fragment shown in Fig. 1B and RNA polymerase isolated from a transition phase culture of M145 (data not shown), providing further evidence that the RNA-protected fragment represented an *in vivo* transcriptional start site. It is preceded by sequences in the –35 (ATGACG) and –10 (CACGAT) regions (Fig. 1B) that show limited similarity to the consensus sequences for the major class of eubacterial promoters (TTGACA and TATAAT, respectively: Hawley and McClure, 1983). This start site lies downstream of the previously assigned translational start codon of *redD* (Narva and Feitelson, 1990); based on these results, as well as FRAME analysis (Bibb *et al.*, 1984) of the *redD* sequence and the similarity of the putative *redD* product to the activators for the actinorhodin and daunorubicin pathways (the products of *act*II–ORF4 (Fernandez-Moreno *et al.*, 1991) and *dnrI* (Stutzman-Engwall *et al.*, 1992), respectively), the GTG located at position 700 (Fig. 1B) probably represents the *redD* translational start codon, yielding a predicted untranslated leader sequence of 199 nucleotides and a protein of 29.6 kDa. The GUG codon would be preceded by a sequence (GGGGG) that is complementary, allowing one G:U base pair, to that found close to the 3' end of the 16S rRNA of *S. coelicolor* A3(2) (CCUCC; (Baylis and Bibb, 1987)), and that is presumed to be involved in ribosome binding.

Analysis of sequences upstream of *redD* had identified the 5' end of an oppositely orientated open reading frame of unknown function that appeared to be transcribed from four promoters, pr1–4 (Narva and Feitelson, 1990; EMBL/GenBank/DDJB Nucleotide Sequence Data Libraries Accession Number M29790). Recent searches of the translated EMBL Nucleotide Sequence Data Library indicate that the product of this gene is homologous (32% and 56% amino acid sequence identity and similarity, respectively, over the available 183-amino-acid

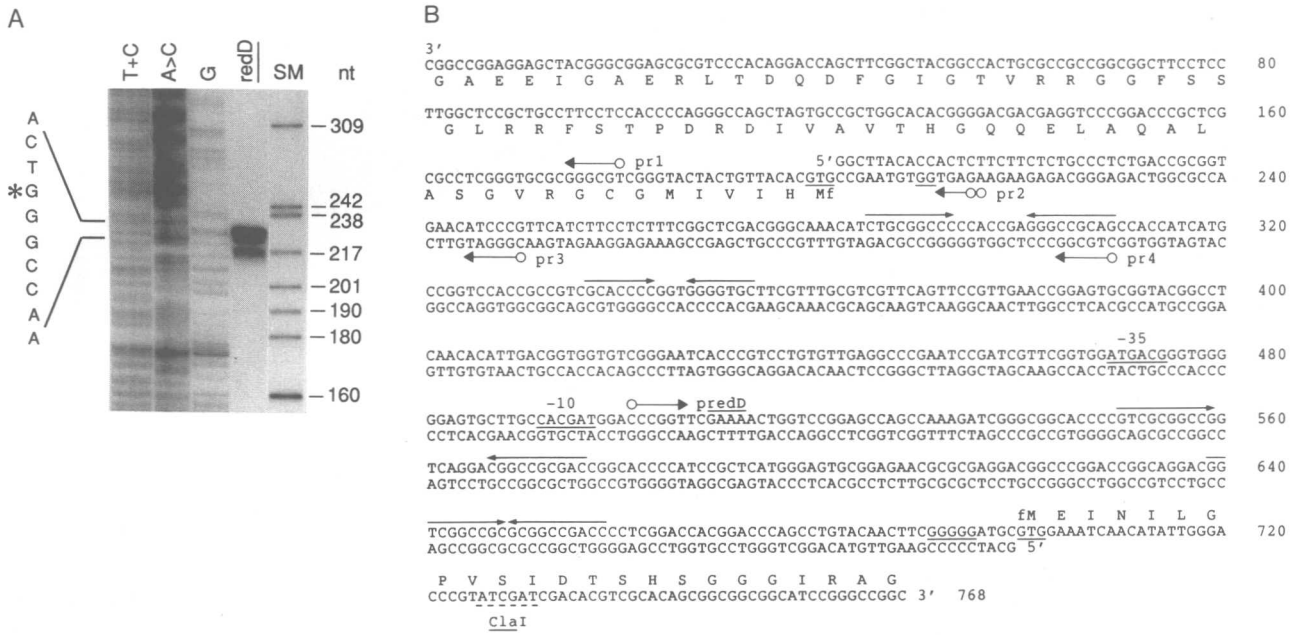

Fig. 1. A. High-resolution S1 nuclease mapping of the transcriptional start site of *redD*. *redD*, the S1-nuclease protected fragment derived from hybridization of the 1.3 kb *NdeI–ClaI* probe uniquely labelled at the 5' end of the *ClaI* site to RNA isolated from a culture producing undecylprodigiosin; T+C, A>C, G are Maxam and Gilbert sequence ladders derived from the same end-labelled fragment; SM, end-labelled *Hpa*II-digested pBR322 size marker. The most likely transcriptional start point is indicated by the asterisk adjacent to the nucleotide sequence of the ladders.
B. Nucleotide sequence of the *redD* promoter region. The sequence of a 768 bp *Nae*I fragment (Narva and Feitelson, 1990) is shown. Probable translational start codons and potential ribosome-binding sites are underlined. The 5' ends of the *redD* mRNA identified in these studies, and of the oppositely orientated transcripts (pr1–4) identified by Narva and Feitelson (1990), are indicated by open circles, and the associated arrows indicate the direction of transcription; putative –10 and –35 regions for *redD* are underlined. Inverted repeats are indicated by converging arrows. The *Cla*I site (5'-AT'CGAT-3') used for S1 nuclease mapping of *redD* is shown at position 726.

sequence) to the product of the *trkA* gene of *E. coli* (EMBL Release 29, Accession Number X52114), which is apparently a membrane protein involved in K^+ transport. Whether the *S. coelicolor* A3(2) gene plays a role in undecylprodigiosin production, or possibly its export, remains to be determined.

Transcriptional activation of redD *and* redX *during the transition from exponential to stationary phase*

To assess the growth-phase expression of *redD* and *redX*, S1 nuclease protection experiments were carried out using RNA from exponential and stationary phase cultures. Although a low level of *redD* transcription was observed during exponential growth, it showed a marked increase during the transition into stationary phase (Fig. 2B); transcription of *redX*, undetectable during exponential growth, occurred on entry into stationary phase, correlating with the appearance of the antibiotic (undecylprodigiosin is strongly hydrophobic and remains cell-associated; Fig. 2C).

ppGpp and transcriptional activation of redD *and* redX *during exponential growth*

ppGpp was detected towards the end of exponential growth of M145, and showed maximal levels on entry into stationary phase, correlating with the appearance of undecylprodigiosin in the culture (Fig. 2A). To assess whether ppGpp might play a role in triggering the onset of antibiotic production, ppGpp production was induced in exponentially growing cultures, either by nutritional shiftdown or by addition of serine hydroxamate, and the effect on *redD* and *redX* transcription was monitored by S1 nuclease mapping. Nutritional shiftdown at an $OD_{450\ nm}$ of about 0.5 led to the production of large intracellular concentrations of ppGpp (a peak level of approximately 200 pmol mg^{-1} dry weight). The culture continued to grow at a much reduced rate, presumably utilizing $(NH_4)_2SO_4$ as nitrogen source, before entering stationary phase 6–7 h after shiftdown (Fig. 3A). No increase in *redD* transcription was detected until 4 h after shiftdown (Fig. 3B), and even then it was considerably less than that observed in a normal growth curve (Fig. 2B). Transcription of *redX* and undecylprodigiosin production were not detected, perhaps because of the lower levels of *redD* transcription. Addition of serine hydroxamate at 25 mM and 50 mM at an $OD_{450\ nm}$ of 0.35 resulted in lower intracellular ppGpp concentrations (peak levels of 47 and 75 pmol mg^{-1} dry weight, respectively) and continued exponential growth, but at much reduced rates (doubling times of 5.1 h and

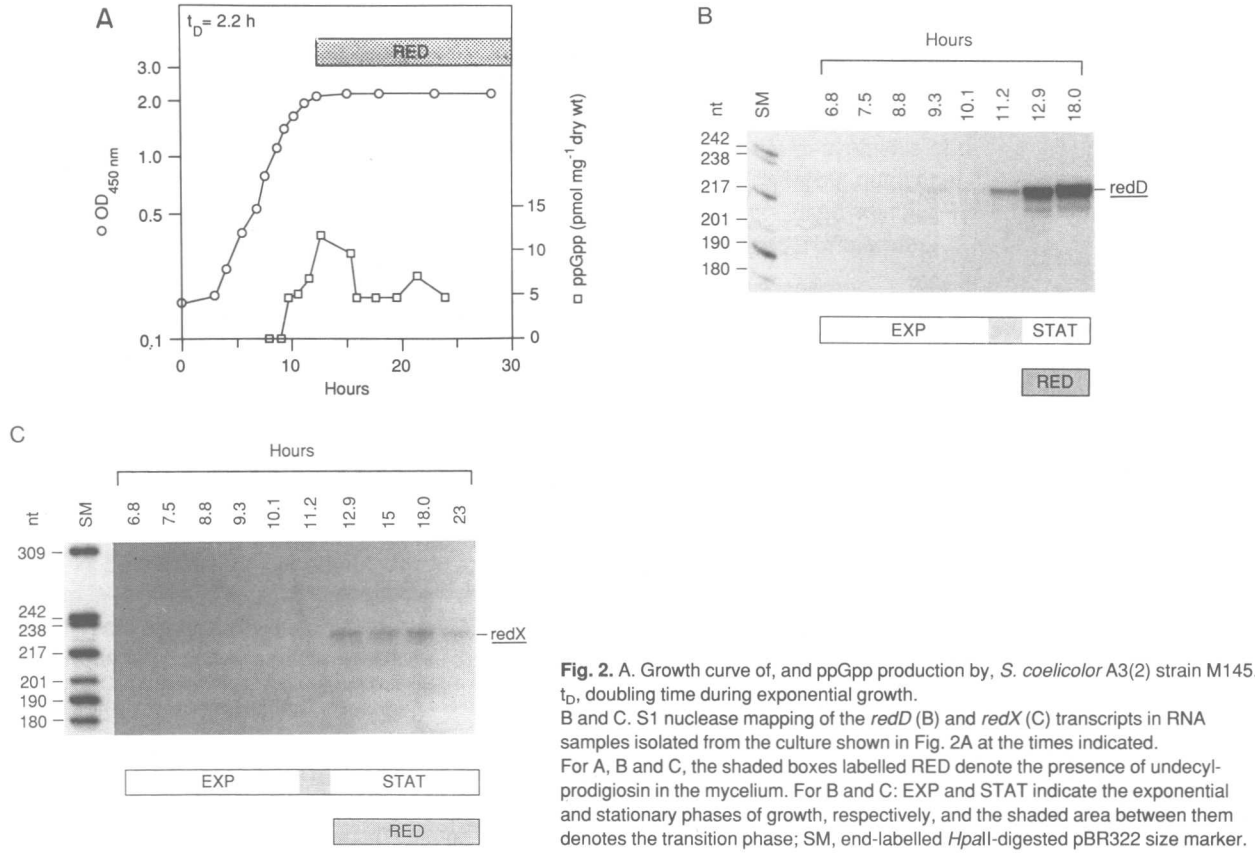

Fig. 2. A. Growth curve of, and ppGpp production by, *S. coelicolor* A3(2) strain M145. t_D, doubling time during exponential growth.
B and C. S1 nuclease mapping of the *redD* (B) and *redX* (C) transcripts in RNA samples isolated from the culture shown in Fig. 2A at the times indicated.
For A, B and C, the shaded boxes labelled RED denote the presence of undecylprodigiosin in the mycelium. For B and C: EXP and STAT indicate the exponential and stationary phases of growth, respectively, and the shaded area between them denotes the transition phase; SM, end-labelled *Hpa*II-digested pBR322 size marker.

17 h, respectively). Transcription of *redD* and *redX*, and undecylprodigiosin synthesis, occurred in the culture treated with 25 mM serine hydroxamate as soon as it entered stationary phase, 11 h after maximal ppGpp production (data not shown).

Multiple copies of redD *result in undecylprodigiosin production during exponential growth*

The stationary phase production of undecylprodigiosin might reflect transcriptional regulation of *redD* alone, or additional limitations imposed on the expression of the biosynthetic structural genes during exponential growth, either at the level of their transcription and/or translation, or by the inhibition or inactivation of the enzymes they encode. To assess this possibility, a 2.4 kb *Bgl*II fragment containing *redD* was cloned into the multicopy (approximately 150 copies per chromosome) plasmid pIJ487 (yielding pIJ6014) in M145, and the effects on transcription of *redD* and *redX*, and on undecylprodigiosin production, were assessed. The growth characteristics of M145(pIJ6014) did not differ significantly from those of M145; however, *redD* transcripts were readily detected at the earliest point in exponential growth from which sufficient RNA could be isolated for analysis, and were present in amounts similar to those observed in stationary phase cultures of M145 (Fig. 4A). The level of *redD* transcript increased during exponential growth and continued to rise in stationary phase. Elevated levels of the *redX* transcript were also observed (Fig. 4B), and undecylprodigiosin was readily detectable in mid-exponential phase. After 65 h, the yield of undecylprodigiosin from M145(pIJ6014) (6.1 μg ml^{-1}) was 5.5 times higher than that from M145 (1.1 μg ml^{-1}).

Discussion

The production of antibiotics in stationary phase, or at low growth rates, might reflect the consumption of a nutrient that represses or inhibits synthesis during growth. We do not believe this to be the case for undecylprodigiosin in these studies for the following reasons. (i) The stationary phase production of undecylprodigiosin in minimal medium severely limited in growth by different carbon sources (e.g. galactose and fructose) strongly suggests the lack of ammonium or phosphate repression and/or

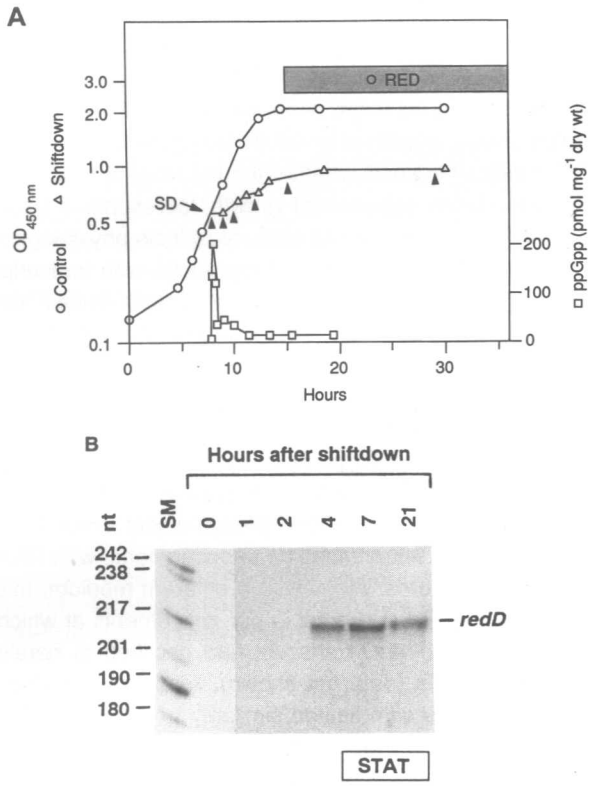

Fig. 3. A. Growth curve of *S. coelicolor* A3(2) strain M145 with (△) and without (○) nutritional shiftdown. The shaded box labelled O RED denotes the presence of undecylprodigiosin in the mycelium of the control culture (○), which grew exponentially with a doubling time of 2.2 h; SD, point of nutritional shiftdown; ▲, time points at which samples were taken from the culture subjected to nutritional shiftdown for RNA isolation.
B. S1 nuclease mapping of the *redD* transcript in RNA samples from the culture subjected to nutritional shiftdown (△ in Fig. 3A) at the times indicated (▲ in Fig. 3A); STAT, stationary phase; SM, end-labelled *Hpa*II-digested pBR322 size marker.

inhibition. (ii) The initial levels of ammonium and phosphate were well below those observed by Hobbs *et al.* (1990) to reduce undecylprodigiosin production significantly. (iii) Since undecylprodigiosin was not made during exponential growth in minimal medium, there is no reason to suspect that supplementation with casamino acids, necessary for studies of nutritional shiftdown, repressed or inhibited production. (iv) Increasing the amount of glucose (fivefold) or phosphate (10-fold) had no significant effect on growth or on the stationary phase onset of undecylprodigiosin production, further suggesting that the latter is not repressed or inhibited by these metabolites at the concentrations normally used. These observations are consistent with the notion that growth rate *per se*, or the cessation of growth, is important in determining the onset of undecylprodigiosin production, at least under these conditions.

Transcription of *redD* in *S. coelicolor* A3(2) M145 increases dramatically as the culture makes the transition from exponential to stationary phase, and transcription of *redX* and the production of undecylprodigiosin clearly occur after growth has finished. This differs from an earlier report (Hobbs *et al.*, 1990) in which undecylprodigiosin synthesis occurred during growth; however, in that work, growth does not appear to have been exponential, but rather was linear, and was much slower than in our experiments, again consistent with a potential role for growth rate in determining the onset of antibiotic biosynthesis. The results obtained with *redX* are similar, although not identical, to those obtained by Guthrie and Chater (1990), who used *redX*::*xylE* fusions to detect

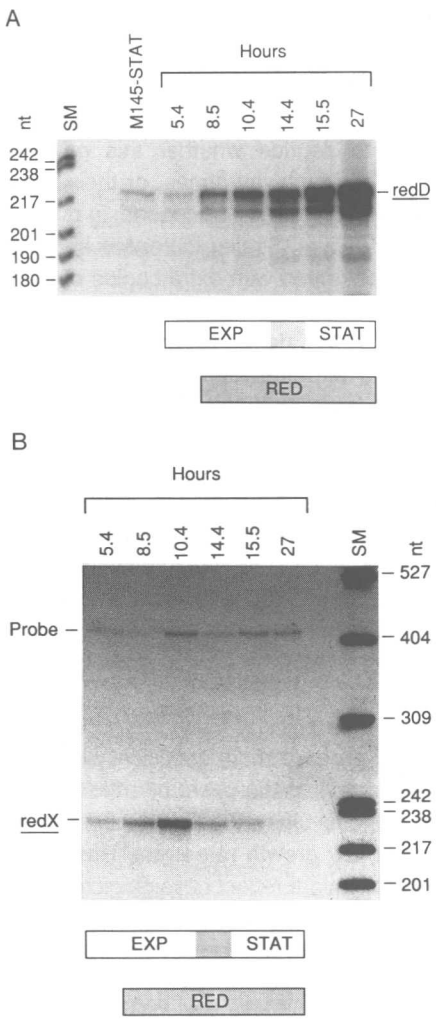

Fig. 4. S1 nuclease mapping of the *redD* (A) and *redX* (B) transcripts in RNA samples isolated from a culture of *S. coelicolor* A3(2) M145 (pIJ6014) at the times indicated. The shaded boxes labelled RED denote the presence of undecylprodigiosin in the mycelium; EXP and STAT indicate the exponential and stationary phases of growth, respectively, and the shaded area between them denotes the transition phase; SM, end-labelled *Hpa*II-digested pBR322 size marker; M145-STAT indicates the use of RNA from a stationary phase culture of M145 (equivalent to the 18 h time point in Fig. 2B)

redX transcription during a relatively long period of transition into stationary phase after inoculating cultures with homogenized mycelium; the use of such inocula, rather than freshly germinated spores, may have led to larger mycelial pellets and therefore to increased physiological heterogeneity, perhaps explaining the departure from exponential growth towards the end of rapid growth (although it should be noted that a different medium was used that might equally be responsible).

Increasing the copy number of *redD* resulted in higher levels of the *redD* and *redX* transcripts, undecylprodigiosin production in exponential growth, and a considerable increase in the yield of the antibiotic. Thus the only limitation to undecylprodigiosin synthesis, at least under these conditions, appears to be the availability of enough of the RedD activator. Increases in undecylprodigiosin production after introduction of cloned copies of *redD* into *S. coelicolor* A3(2) were noted also by Malpartida *et al.* (1990) and Narva and Feitelson (1990), although it was not possible to deduce whether this reflected earlier expression of the pathway genes, or their expression at higher levels once growth had ceased; in our studies, the former clearly occurs. Similar increases in antibiotic production were observed with extra copies of the pathway-specific activator genes for actinorhodin (Fernandez-Moreno *et al.*, 1991), daunorubicin (Stutzman-Engwall *et al.*, 1992), and streptomycin (Ohnuki *et al.*, 1985); as noted by Chater (1990), extension of this principle to industrial fermentations could potentially achieve equally dramatic improvements in antibiotic titre, particularly with unimproved strains. The reason for the continuous increase in the level of the *redD* transcript during growth of M145(pIJ6014) (Fig. 4A) is unknown, but may reflect a possible increase in plasmid copy number, or possibly changes in supercoiling that might result in elevated levels of expression of the now plasmid-borne *redD* promoter.

Our results are consistent with the notion of a role for growth rate, or perhaps more specifically the cessation of growth, in determining the onset of antibiotic production. Although antibiotic biosynthesis has been observed in chemostats at low growth rates (see Bushell (1989) for references), this might reflect physiological heterogeneity within mycelial clumps, and production by cells in the interior that have stopped growing. In this context, it is interesting to note the apparent ability of ppGpp to reduce growth rate in *Escherichia coli* (Sarubbi *et al.*, 1988; Schreiber *et al.*, 1991), and the apparent correlation of ppGpp and antibiotic biosynthesis, albeit incomplete, previously observed in several streptomycetes (Ochi, 1986; 1987a,b; 1990; Strauch *et al.*, 1991; Kelly *et al.*, 1991). Although we saw a correlation between ppGpp synthesis and transcription of *redD* during the transition of a normal culture into stationary phase, this was not observed after nutritional shiftdown or after addition of serine hydroxamate. These results, and the observations of Bascarán *et al.* (1991), suggest that an increase in the level of ppGpp is not a sufficient physiological signal for the activation of transcription of antibiotic biosynthetic genes.

It is difficult to know whether the low level of *redD* transcription during exponential growth represents a basal level of expression from all cells, or reflects physiological heterogeneity within clumps of mycelium, with transcription exclusively from those that are growth-limited; the results obtained with the multi-copy construct pIJ6014 are consistent with either interpretation. Although the elevated levels of the *redD* transcript observed with this multi copy plasmid might reflect titration of a putative *redD* repressor, there is no genetic evidence to suggest the existence of a negatively acting regulatory gene.

Narva and Feitelson (1990) failed to detect a *redD* transcript by S1 nuclease mapping in experiments with RNA from 2–3 d cultures. Albeit with a different medium, this would correspond to a point in our experiments at which the level of the *redD* transcript had declined to barely detectable levels (data not shown). *redD* is transcribed from a promoter with limited similarity to the consensus sequence for the major class of eubacterial promoters. Although the preliminary experiments with RNA polymerase might indicate a role for a minor holoenzyme containing an alternative sigma factor in *redD* activation (see Buttner (1989) for a review of RNA polymerase heterogeneity in *Streptomyces*), the transcripts we observed *in vitro* might represent a basal level of *redD* transcription by the major holoenzyme in the absence of a positively acting regulatory molecule that might be required for elevated activity *in vivo*.

redD is homologous to the pathway-specific activator gene (*actII–ORF4*) required for actinorhodin biosynthesis in the same host (Fernandez-Moreno *et al.*, 1991), to the activator gene (*dnrI*) for daunorubicin production in *Streptomyces peucetius* (Stutzman-Engwall *et al.*, 1992), and to the 5' end of the putative pleiotropic regulatory gene *afsR* of *S. coelicolor* A3(2) (Horinouchi *et al.*, 1990). Alignment of the amino acid sequences they encode fails to reveal a convincing DNA-binding motif that is common to all four proteins, and previously suggested possible motifs (Stutzman-Engwall *et al.*, 1992; Horinouchi *et al.*, 1990) fall outside the regions of homology; perhaps these genes encode a family of regulatory proteins with a novel means of recognizing specific nucleotide sequences.

Experimental procedures

Bacterial strains and culture conditions

High-density spore preparations (about 10^{10} colony-forming units ml^{-1}) of *S. coelicolor* A3(2) strain M145 ($SCP1^-$, $SCP2^-$,

prototrophic) were obtained and pre-germinated as previously described (Strauch *et al.*, 1991). Aggregated germlings were dispersed by brief sonication and inoculated into 50 ml of medium in 250 ml siliconized flasks containing coiled stainless steel springs (about 25 cm flask^{-1}, with 2.5 turns cm^{-1}). Unless otherwise stated, the medium contained 55 mM (1% w/v) glucose, 25 mM TES buffer (pH 7.2), 15 mM $(NH_4)_2SO_4$, 0.5 mM NaH_2PO_4, 0.5 mM K_2HPO_4, 5 mM $MgSO_4$, 5% w/v PEG 6000, 1% v/v Rhodorsil Antimousse 426R antifoam, and 0.1% v/v trace element solution (0.1 g l^{-1} of each of $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CaCl_2.6H_2O$ and NaCl)) supplemented with 0.2% w/v casamino acids. The inoculum was adjusted to give an $OD_{450\ nm}$ against water of about 0.1 (0.05 of which reflected medium components (largely antifoam)); this corresponded to a spore inoculum of 4×10^6 ml^{-1}. Flasks were incubated at 30°C and 300 r.p.m., and growth was monitored at $OD_{450\ nm}$ against water. The conditions used were modified from those adopted and developed by Hodgson (1982); the high spore inoculum, coiled springs and PEG 6000 all contributed to dispersed growth. Nutritional shiftdown, treatment with serine hydroxamate, and assessment of undecylprodigiosin production were performed as previously described (Strauch *et al.*, 1991).

Plasmids

For analysis of *redD* transcription, a 2.4 kb *Bam*HI–*Pst*I fragment from pIJ2341 (Malpartida *et al.*, 1990) that contains all of *redD* (Narva and Feitelson, 1990) was cloned in *Bam*HI–*Pst*I-cleaved pIJ2926 (a derivative of pUC18 (Yanisch-Perron *et al.*, 1985), with *Bgl*II sites flanking a modified polylinker; G. R. Janssen, personal communication) to yield pIJ6013. For analysis of *redX* transcription, the 1.4 kb *Bam*HI fragment from pIJ2341 that is internal to a *red* biosynthetic transcript (Malpartida *et al.*, 1990; Guthrie and Chater, 1990) was cloned in pSPT18 (obtained from Boehringer Mannheim) with the promoter-proximal *Bam*HI site adjacent to the SP6 promoter of the vector; the resulting plasmid (pIJ6000) was subjected to restriction mapping and a *Sma*I site was found approximately 220 bp from the promoter-proximal end of the insert. pIJ6014 is a derivative of pIJ487 (Ward *et al.*, 1986) with the 2.4 kb *Bgl*II *redD* fragment from pIJ6013 inserted in the *Bgl*II site of the polylinker.

S1 nuclease mapping

For each S1 nuclease reaction, 30 μg of RNA was hybridized in NaTCA buffer (Murray, 1986) to about 0.02 pmol (approximately 10^4 Cerenkov c min^{-1}) of labelled probe. For *redD*, a 1.3 kb *Cla*I–*Nde*I fragment from pIJ6013 that contains the *redD* promoter region was uniquely labelled with ^{32}P (Maxam and Gilbert, 1980) at the 5' end of the *Cla*I site within the *redD* coding region (Fig. 1B, nucleotide position 726) and used as probe. The results reported were confirmed using a probe uniquely end-labelled at a position 147 nucleotides downstream of the *redD* translational start codon and generated using the polymerase chain reaction (Ehrlich, 1989) and synthetic oligonucleotides; no additional transcriptional start sites were detected. For *redX*, a 990 bp *Sma*I–*Aat*II fragment from pIJ6000 uniquely labelled (Maxam and Gilbert, 1980) at the 5' end of the *Sma*I site that lies an undetermined distance downstream of the *redX* promoter was used as probe. All subsequent steps were as described in Strauch *et al.*, (1991). Nucleotide sequence ladders were derived as described by Maxam and Gilbert (1980). Before assigning a precise RNA initiation site for *redD*, one nucleotide was subtracted from the length of the protected fragment to account for the difference in 3' ends resulting from S1 nuclease digestion and the chemical sequencing reactions (Hentschel *et al.*, 1980).

Amino acid sequence comparisons

The amino acid sequences encoded by *redD* and by the 5' end of the oppositely orientated open reading frame were used to search Release 29 of the EMBL Data Library using TFASTA; alignments of the *redD* product with those of *act*II–ORF4, *dnrI* and *afsR*, and of the protein encoded by the oppositely oriented open reading frame with the *trkA* product, were achieved using BESTFIT; both programs were accessed via the UWGCG Package (Devereux *et al.*, 1984).

Reproducibility

Each experiment was performed in its entirety at least twice, and usually three times. The results shown in this paper were typical of the repeated experiments.

Acknowledgements

We thank Paco Malpartida for the gift of pIJ2341, Mark Buttner, Keith Chater, David Hopwood and Tobias Kieser for their comments on the manuscript, and David Hodgson for many useful conversations. The work was supported by grant-in-aid from the Agricultural and Food Research Council and the John Innes Foundation. E.S., H.C.G. and N.A. were recipients of postdoctoral fellowships from the Deutscher Akademischer Austauschdienst, CONICET, and The Royal Society, respectively.

References

Bascarán, V., Sánchez, L., Hardisson, C., and Braña, A.F. (1991) Stringent response and initiation of secondary metabolism in *Streptomyces clavuligerus*. *J Gen Microbiol* **137:** 1625–1634.

Baylis, H.A., and Bibb, M.J. (1987) The nucleotide sequence of a 16S rRNA gene from *Streptomyces coelicolor* A3(2). *Nucl Acids Res* **15:** 1716.

Bibb, M.J., Findlay, P.R., and Johnson, M.W. (1984) The relationship between base composition and codon usage in bacterial genes and its use in the simple and reliable identification of protein coding sequences. *Gene* **30:** 157–166.

Bushell, M.E. (1989) The process physiology of secondary metabolite production. In *Microbial Products: New Approaches*. Baumberg, S., Hunter, I., and Rhodes, M. (eds). Cambridge: Cambridge University Press, pp. 95–120.

Buttner, M.J. (1989) RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **3:** 1653–1659.

Chater, K.F. (1989) Aspects of multicellular differentiation in *Streptomyces coelicolor* A3(2). In *Genetics and Molecular Biology of Industrial Microorganisms*. Hershberger, C.L., Queener, S.W., and Hegeman, G. (eds). Washington D.C.: American Society for Microbiology, pp. 99–107.

Chater, K.F. (1990) The improving prospects for yield increase by genetic engineering in antibiotic-producing streptomycetes. *Bio/Technology* **8:** 115–121.

Coco, E.A., Narva, K.E., and Feitelson, J.S. (1991) New classes of *Streptomyces coelicolor* A3(2) mutants blocked in undecylprodigiosin (Red) biosynthesis. *Mol Gen Genet* **227:** 28–32.

Demain, A.L. (1989) Carbon source regulation of idiolite biosynthesis in actinomycetes. In *Regulation of Secondary Metabolism in Actinomycetes.* Shapiro, S. (ed.). Boca Raton: CRC Press, pp. 127–134.

Demain, A.L., Aharonowitz, Y., and Martin, J.-F. (1983) Metabolic control of secondary biosynthetic pathways. In *Biochemistry and Genetic Regulation of Commercially Important Antibiotics.* Vining, L.C. (ed.). London: Addison-Wesley, pp. 49–72.

Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucl Acids Res* **12:** 387–395.

Ehrlich, H.A. (1989) *PCR Technology.* New York: Stockton Press.

Feitelson, J.S., Malpartida, F., and Hopwood, D.A. (1985) Genetic and biochemical characterization of the *red* gene cluster of *Streptomyces coelicolor* A3(2). *J Gen Microbiol* **131:** 2431–2441.

Fernandez-Moreno, M.A., Caballero, J.L., Hopwood, D.A., and Malpartida, F. (1991) The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* transfer RNA gene of *Streptomyces. Cell* **66:** 769–780.

Guthrie, E.P., and Chater, K.F. (1990) The level of a transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a transfer RNA Gene. *J Bacteriol* **172:** 6189–6193.

Hallam, S.E., Malpartida, F., and Hopwood, D.A. (1988) Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor. Gene* **74:** 305–320.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucl Acids Res* **11:** 2237–2255.

Hentschel, C., Irminger, J.-C., Bucher, P., and Birnsteil, M.L. (1980) Sea urchin histone mRNA termini are located in gene regions downstream from putative regulatory sequences. *Nature* **285:** 147–151.

Hobbs, G., Frazer, C.M., Gardner, D.C.J., Flett, F., and Oliver, S.G. (1990) Pigmented antibiotic production by *Streptomyces coelicolor* A3(2) — kinetics and the influence of nutrients. *J Gen Microbiol* **136:** 2291–2296.

Hodgson, D.A. (1982) Glucose repression of carbon source uptake in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. *J Gen Microbiol* **128:** 2417–2430.

Hopwood, D.A. (1988) Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. *Proc R Soc Lond* B **235:** 121–138.

Horinouchi, S., Kito, M., Nishiyama, M., Furuya, K., Hong, S.K., Miyake, K., and Beppu, T. (1990) Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). *Gene* **95:** 49–56.

Kelly, K.S., Ochi, K., and Jones, G.H. (1991) Pleiotropic effects of a *relC* mutation in *Streptomyces antibioticus. J Bacteriol* **173:** 2297–2300.

Malpartida, F., Niemi, J., Navarrete, R., and Hopwood, D.A. (1990) Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. *Gene* **93:** 91–99.

Maxam, A.M., and Gilbert, W. (1980) Sequencing end-labelled DNA with base specific chemical cleavages. *Meth Enzymol* **65:** 449–560.

Murray, M.G. (1986) Use of sodium trichloroacetate and mung bean nuclease to increase sensitivity and precision during transcript mapping. *Anal Biochem* **158:** 165–170.

Narva, K.E., and Feitelson, J.S. (1990) Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). *J Bacteriol* **172:** 326–333.

Ochi, K. (1986) Occurrence of the stringent response in *Streptomyces* sp. and its significance for the initiation of morphological and physiological differentiation. *J Gen Microbiol* **132:** 2621–2631.

Ochi, K. (1987a) A *rel* mutation abolishes the enzyme induction needed for actinomycin synthesis by *Streptomyces antibioticus. Agric Biol Chem* **51:** 829–835.

Ochi, K. (1987b) Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: Significance of the stringent response (ppGpp) and GTP content in relation to A factor. *J Bacteriol* **169:** 3608–3616.

Ochi, K. (1990) A relaxed (*rel*) mutant of *Streptomyces coelicolor* A3(2) with a missing ribosomal protein lacks the ability to accumulate ppGpp, A-factor and prodigiosin. *J Gen Microbiol* **136:** 2405–2412.

Ohnuki, T., Imanaka, T., and Aiba, S. (1985) Self cloning in *Streptomyces griseus* of a *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. *J Bacteriol* **164:** 85–94.

Rudd, B.A.M., and Hopwood, D.A. (1980) A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal gene cluster. *J Gen Microbiol* **119:** 333–340.

Sarubbi, E., Rudd, K.E., and Cashel, M. (1988) Basal ppGpp level adjustment shown by new *spoT* mutants affect steady state growth rates and *rrnA* ribosomal promoter regulation in *Escherichia coli. Mol Gen Genet* **213:** 214–222.

Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M., and Glaser, G. (1991) Overexpression of the *relA* gene in *Escherichia coli. J Biol Chem* **266:** 3760–3767.

Strauch, E., Takano, E., Baylis, H.A., and Bibb, M.J. (1991) The stringent response in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **5:** 289–298.

Stutzman-Engwall, K.J., Otten, S., and Hutchinson, C.R. (1992) Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius. J Bacteriol* **174:** 144–154.

Tsao, S.W., Rudd, B.A.M., He, X., Chang, C., and Floss, H.G. (1985) Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. *J Antibiot* **38:** 128–130.

Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J., Buttner, M.J., and Bibb, M.J. (1986) Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. *Mol Gen Genet* **203:** 468–478.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119